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Monitor for Chemical Carcinogens

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HEMOGLOBIN BINDING AS A DOSE MONITOR FOR CHEMICAL CARCINOGENS

By

Michael A. Pereira and Lina W. Chang

U.S. ENVIRONMENTAL PROTECTION AGENCY
HEALTH EFFECTS RESEARCH LABORATORY
26 WEST ST. CLAIR STREET
CINCINNATI, OHIO 45268
TELEPHONE NO. 513/684-7411

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16. ABSTRACT The covalent binding of chemical carcinogens and mutagens to hemoglobin has been proposed as a dose monitor for environmental exposure. The binding of chloroform and bromoform to hemoglobin in rats was demonstrated to result from the formation of adducts to amino acids in the globin. The altered amino acids were isolated with an amino acid analyzer employing ion exchange chromatography. At low doses the covalent binding of chloroform, and 2-acetylaminofluorene, were linearly related to dose. At higher doses, the binding increased as a non-linear and decreased function of dose. A pharmacokinetic model was described that relates the dose as determined by hemoglobin binding, to exposure, and carcinogenic potency. The quantitative difference of carcinogenic potencies for chemical carcinogens was not reflected to the Hemoglobin Binding Indices of the carcinogens. Therefore, the binding of a carcinogen to hemoglobin appears to be suitable for use in the estimation of the systemic dose and exposure to the carcinogen but not for the estimation of the carcinogenic potency.		
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FIGURES

- Figure 1: Pharmacokinetic model of hemoglobin alkylation. The pharmacokinetic model of hemoglobin alkylation by environmental chemicals can be used to estimate the concentration of reactive metabolic (RM) of carcinogens. The estimated concentration of RM can then be used to derive estimates of dose, exposure and target organ macromolecular binding. RM-DNA, and RM-Hb represents the reactive metabolite covalently bound to DNA and hemoglobin respectively.
- Figure 2: Time course of the binding of chloroform -[^{14}C] to rat hemoglobin. Rats were administered orally 10 $\mu\text{mol/kg}$ chloroform - [^{14}C] and sacrificed at various times later. Results are averages from 6 rats + S.E.
- Figure 3: Dose dependency of 2-acetylaminofluorene -[^{14}C] and chloroform -[^{14}C] bound to rat hemoglobin. Rats were administered orally various doses of 2-acetylaminofluorene -[^{14}C] or chloroform -[^{14}C] and sacrificed 24 hours later. Results are averages from 6 rats + S.E. The line with a slope of 1 that best fits the data is shown.

Figure 4:

Bio-Rex 70 column chromatography elution profile of a: chloroform -[^{14}C] bound in vitro to hemoglobin, b: chloroform - [^{14}C] bound in vivo to hemoglobin and c: control hemoglobin. Protein was determined by the Bradford (1976) procedure.

Figure 5:

Amino acid analysis of the acid hydrolyzate of chloroform -[^{14}C] bound in vitro to hemoglobin. Amino acid analysis was performed with a Beckman 118 BL. Tritium labeled amino acids were added as internal standards to the hydrolyzate.

The estimation from animal bioassay data of the carcinogenic risk to humans resulting from an exposure to a chemical carcinogen requires knowledge of the dose and pharmacokinetics of the chemical in humans. Most of the other assays described at this conference such as mutagenicity of body fluids, unscheduled DNA synthesis, alkaline elution, cytogenetics, sister chromatid exchange, micronuclei and lymphocyte mutagenesis are indicators of an exposure to a mutagen or tumor initiator but are not dose monitors. They do not give a quantitative estimate of the dose received. The systemic dose in humans has to be obtained by quantitation of the carcinogen and metabolites in such body fluids as blood, urine, sputum, semen and milk and feces. Target organ dose in most cases can not be obtained but has to be estimated from the systemic dose.

A pharmacokinetic model is outlined in Figure 1 that relates the systemic dose by way of target organ dose to carcinogenic potency (Pereira et al 1981). Two types of tumor initiators have been identified; direct acting initiators are electrophiles capable of binding macromolecules, while indirect acting initiators require metabolic activation to an electrophile (RM) before they can bind. If RM is not detoxified, it can bind to protein, lipid, RNA or DNA. The binding of RM to lipid and protein can result in cytotoxicity. The binding to DNA results in the formation of altered bases that can 1) be repaired, 2) be innocuous or 3) result in mispairing of the altered base during DNA replication. The alteration in the genome is then fixed and transmitted onto daughter cells. A critical alteration in the genome can result in a transformed cell and thus initiate the carcinogenic progression.

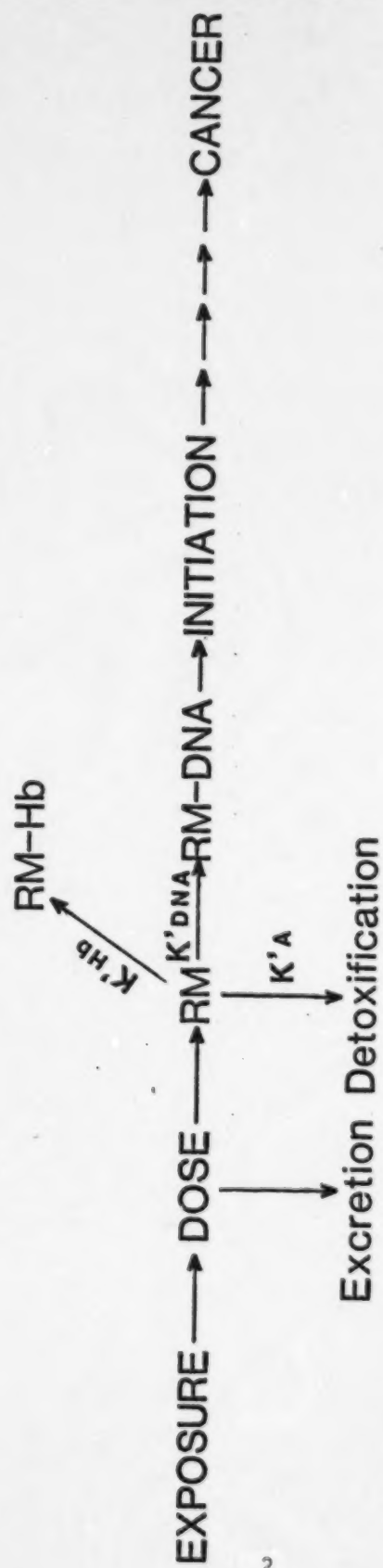


Figure 1.

Of major importance in the extrapolation of animal data to estimate human response, is a knowledge of species variation in metabolism to RM and in detoxification. Estimates of human metabolism can be obtained from accidentally exposed people by measurement of metabolites in body fluids, feces and expired air. A major drawback for measuring the presence of metabolites in humans is that the analyses must be performed immediately, that is, prior to the excretion of the metabolites. The binding of RM to two different macromolecules in blood i.e. hemoglobin and lymphocyte DNA, have been proposed as surrogates for estimating RM (Figure 1). Neither type of binding directly results in carcinogenesis but is rather an indirect measure of RM, and can be used to estimate species variation in metabolism, systemic dose and carcinogenic activity as depicted schematically by the pharmacokinetic model in Figure 1.

Chloroform has been demonstrated to induce hepatocellular carcinomas in mice and kidney epithelial tumors in male rats (National Cancer Institute, 1976). Chemicals can increase the incidence of cancer by two distinct mechanisms: genetic and epigenetic (Boutwell, 1974, 1978). Chloroform does not appear to be a genotoxic carcinogen. Chloroform was non-mutagenic in 1) the Ames Salmonella/microsome assay (Simmon et al., 1977), 2) E. Coli K 12 for basepair substitution (Uehleke et al, 1976) and 3) 8-azaguanine locus in Chinese hamster lung fibroblast in cell culture (Sturrock, 1977). The amount of covalent binding of chloroform to rat liver and kidney DNA was very minimal compared to other carcinogens (Reitz et al, 1980; Pereira et al, 1981). The negative evidence for chloroform mutagenicity and the low level of DNA binding has resulted in the proposal that chloroform is an epigenetic carcinogen.

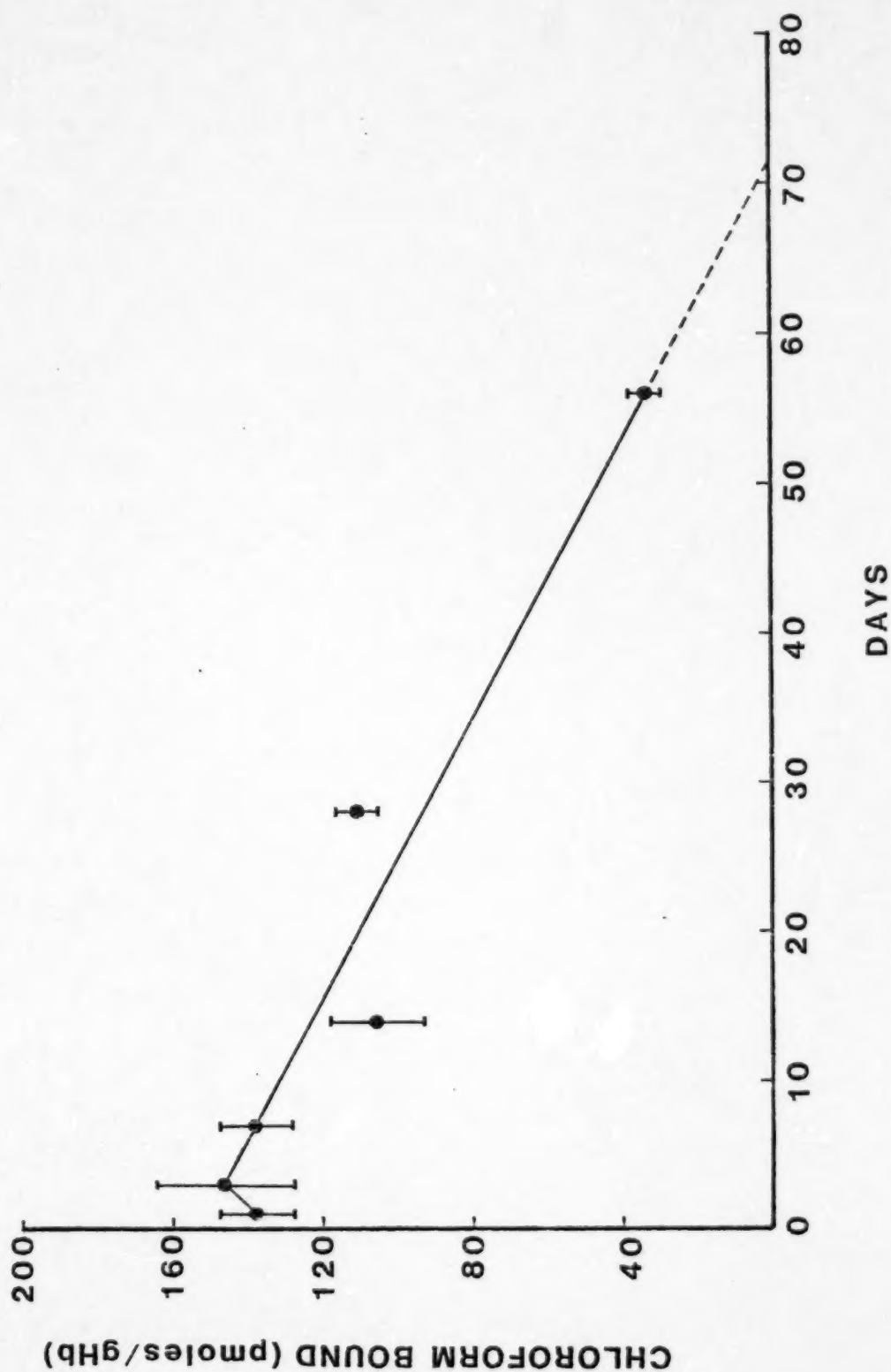


Figure 2.

Therefore, the indicators of exposure that are based on mutagenesis including binding to lymphocyte DNA are not appropriate for demonstrating human exposure to epigenetic carcinogens such as chloroform.

Humans are exposed to ppb levels of chloroform in their drinking water (Symons et al 1975). The chloroform is produced during chlorination by the reaction of chlorine with the organics including the fulvic and humic acids present in the water. Chloroform has been demonstrated to bind covalently to protein (Reynolds and Yee, 1967; Ilett et al, 1973; Hill et al, 1975; Uehleke and Werner, 1975). Therefore, the binding to hemoglobin of epigenetic carcinogens such as chloroform which covalently bind protein might be an appropriate dose monitor.

STABILITY OF HEMOGLOBIN BINDING AND DOSE-RESPONSE RELATIONSHIPS

The major advantages of hemoglobin binding as a dose monitor are 1) easy access from humans in large quantities (five ml of blood yield 0.5 to 0.75 gm hemoglobin) and 2) circulating hemoglobin is stable with a life time of 45-68 and 120 days in rats and humans respectively. The life time of hemoglobin containing covalently bound metabolites of chloroform as shown in Figure 2, was determined in rats to be approximately 70 days which is (approximately the life time of rat erythrocytes (Pereira and Chang, 1982). Over 70% of the radioactivity in these preparations was determined to be amino acid adducts and not to represent metabolic incorporation into de novo synthesis of hemoglobin. The shape of the life time curve indicates that hemoglobin of all ages contained bound metabolite of chloroform. This is similar to the data reported by Ehrenberg (1979) at the first Banbury Conference for the binding of dimethylnitrosamine in mice. We have also shown that the extent of

chloroform binding to hemoglobin resulting from 10 daily doses equalled the binding that resulted from a single dose equivalent to the total of the 10 daily doses. Therefore, the binding of a carcinogen to hemoglobin can be used to integrate the dose obtained during the long life-time of circulating hemoglobin so that low chronic human exposures might be detected even if the daily exposure was below detection. This is a major advantage that hemoglobin binding has over measurement of metabolites in body fluids and lymphocyte DNA binding (DNA repair could prevent the accumulation of adducts to bases).

A list of the chemical carcinogens that we have shown to bind hemoglobin when administered orally, is presented in Table 1. Other carcinogens and mutagens that bind hemoglobin include ethylene oxide (Ehrenberg et al, 1974; Osterman-Golkar et al. 1976) and vinyl chloride (Osterman-Golkar et al 1977). Both direct and indirect acting carcinogens bound hemoglobin. The direct acting carcinogens in general bound hemoglobin to a greater extent than indirect acting carcinogens. Much of the RM of indirect acting carcinogens formed presumably in the liver was probably detoxified, hydrolyzed and bound to other macromolecules before it reached the circulating erythrocytes to bind hemoglobin. This might explain why weak carcinogens that are direct acting alkylating agents bind hemoglobin to a greater extent than potent indirect acting carcinogens. There was no relationship among the indirect acting carcinogens between the extent of binding to hemoglobin and carcinogenic potency. For example, aflatoxin B₁ the most potent carcinogen tested was eighth in the order of binding among the indirect acting carcinogens and benzo(a)pyrene bound to a greater extent than 7,12-dimethylbenz(a)anthracene. Differences in the stability of the RMs of the carcinogens and in their

TABLE 1
CARCINOGENS THAT IN VIVO COVALENTLY BIND HEMOGLOBIN^a

Chemical	Binding Index ^b
<u>A. Direct Acting Carcinogens</u>	
1. Methyl Methanesulphonate	3320
2. N-Nitroso-Methylurea	302
3. N-Nitroso-Ethylurea	217
4. Methyl-N-Nitro-N-Nitrosoguanidine	180
<u>B. Indirect Acting Carcinogens</u>	
1. Dimethylnitrosamine	697
2. Benzo(a)pyrene	181
3. 3-Methylcholanthrene	165
4. Diethylnitrosamine	153
5. Benzene	144
6. 2-Acetylaminofluorene	108
7. 7,12-Dimethylbenz(a)anthracene	99.8
8. Aflatoxin B ₁	39.4
9. Aniline	24.8
10. Benzidine	22.5
11. Chloroform	14.2
12. Carbon Tetrachloride	10.2

^aPereira and Chang 1981.

^bBinding Index: pmole bound/g hemoglobin/umole/kg bd wt

ability to reach circulating erythrocytes as well as differences in the relationship of the RMs to carcinogenic potency are reasons why hemoglobin binding can not be used to rank carcinogens according to their potency.

The dose-response relationship of the binding of chloroform and 2-acetylaminofluorene to hemoglobin is shown in Figure 3. The extent of chloroform and 2-acetylaminofluorene binding to hemoglobin was linearly dependent upon dose between 0.1 and 100 umoles/kg (Pereira et al 1981; Pereira and Chang, 1982). Above 100 umole/kg chloroform the binding increased at a reduced rate. The binding of 2-acetylaminofluorene to liver DNA was also related to dose in a linear manner from 1 to 100 umole/kg (Pereira et al, 1981). However, the binding of 2-acetylaminofluorene to hemoglobin and DNA did not support, as proposed in Figure 1, a quantitative relationship between macromolecular binding and carcinogenic potency. The lack of a quantitative relationship between target organ dose measured either as DNA binding or indirectly as hemoglobin binding might be due to the many confounding steps in the neoplastic progression between initiation and cancer.

ISOLATION OF CARCINOGEN ADDUCTS TO HEMOGLOBIN

Isolation of hemoglobin containing carcinogen adducts:

Microsomes (109,000xg for 1 hour) were isolated from Aroclor 1254 induced Sprague-Dawley rats (Charles River, Portage, MI). The microsomes were suspended in 0.05M KPO₄ (pH 7.5). The incubation medium contained 1 gm rat hemoglobin, 4.4mCi chloroform ¹⁴C (ICN Pharmaceuticals, Inc., Irvin, CA; 10 mCi/mmol; Lot No.: 981492). 0.4 mmole MgCl₂, 1 mmole KPO₄ (pH 7.0), 0.128 mmole glucose-6-phosphate, 40 mg NADPH, 500 units glucose-6-phosphate dehydrogenase and 1 mg microsomal proteins in a total volume of 40 ml. The

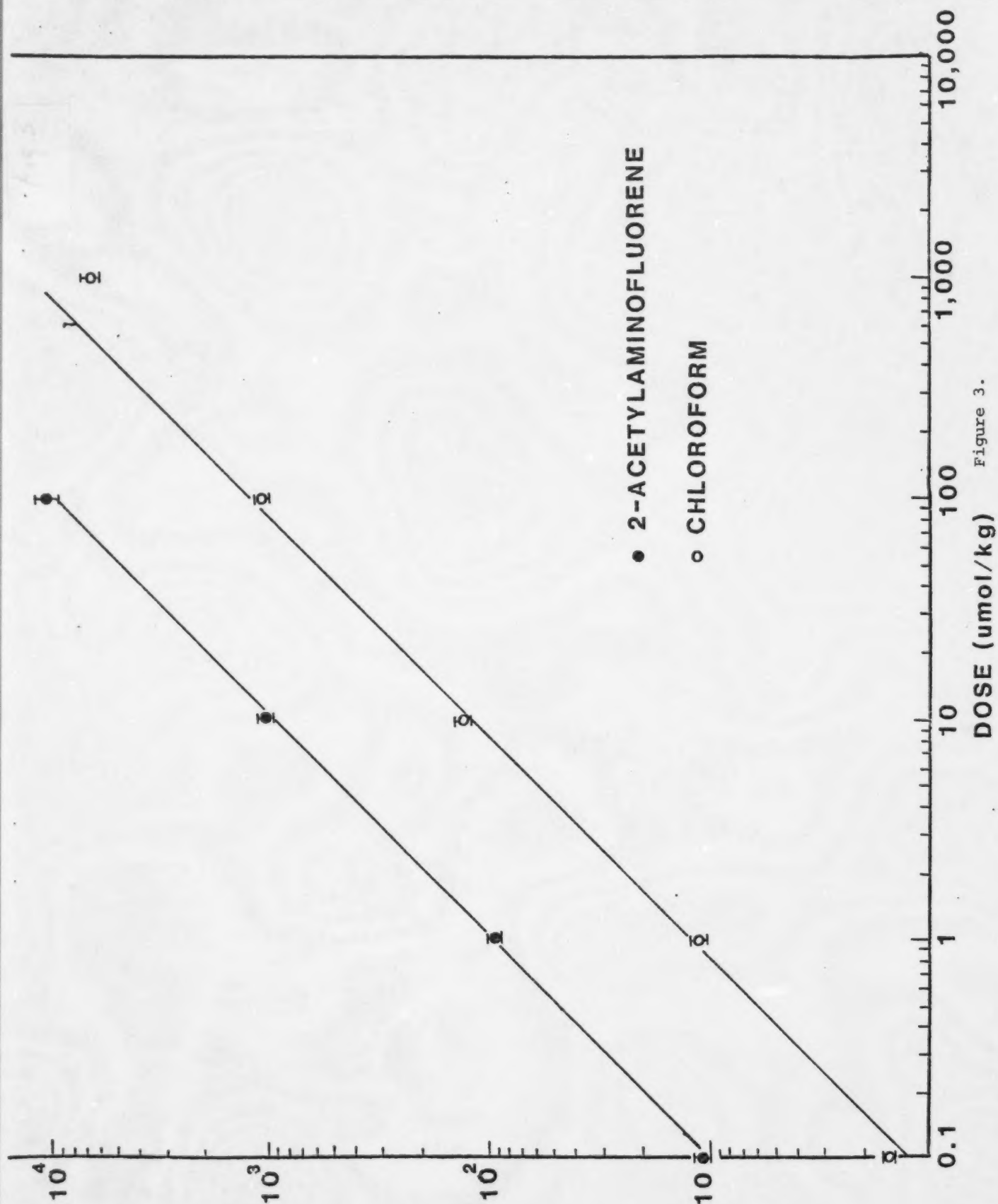


Figure 3.

incubation was performed under oxygen at 37°C for two hours. An aliquot (0.5 ml) of the incubation was diluted with 1 ml water and extracted 3 times with 1 ml toluene. The aqueous phase was saturated with carbon monoxide, centrifuged and 0.5-1ml applied to a Bio-Rex 70 column (0.9 X 11 cm; Bio-Rad Laboratories, Richmond, CA). The column was eluted sequentially with Developer 4 (pH 6.91, 50 mM Na⁺) and high phosphate buffer (pH 6.37, 392 mM Na⁺) as described by Cole et al, 1978. A high specific activity peak I (4.4×10^5 dpm/protein) was eluted early in Developer 4 (Figure 4a). A much lower specific activity peak II (8.9×10^4 dpm/mg protein) was eluted in the high phosphate buffer. Peak I represented a 4.9 fold enrichment of the hemoglobin containing chloroform derived adducts. Similar enrichment of the hemoglobin containing bound carcinogen has also been obtained in a peak eluted by Developer 4 for in vivo bound chloroform (Figure 4b). Hemoglobin from rats not treated with carcinogens contained very little, if any, of the peak eluted by Developer 4 (Figure 4c). The separation of hemoglobin containing bound carcinogen by Bio-Rex 70 chromatography should prove very useful as a first step in the purification of the carcinogen adducts to the amino acids in hemoglobin.

Isolation of Chloroform Adduct:

Peak I of the Bio-Rex 70 elution containing the in vitro bound chloroform-¹⁴ (was reduced with sodium borohydrate and desalted by dialysis. The globin was precipitated with acetone containing 1% HCl (-20°C) and hydrolyzed with 6N HCl as previously described (Pereira and Chang, 1982). The hydrolyzate was evaporated to dryness, dissolved in 0.2N sodium citrate (pH 2.2) and analyzed in a Beckman 118 BL amino acid analyzer equipped with a stream divider (Beckman Instrument Inc. Palo Alto, CA). The radioactivity was

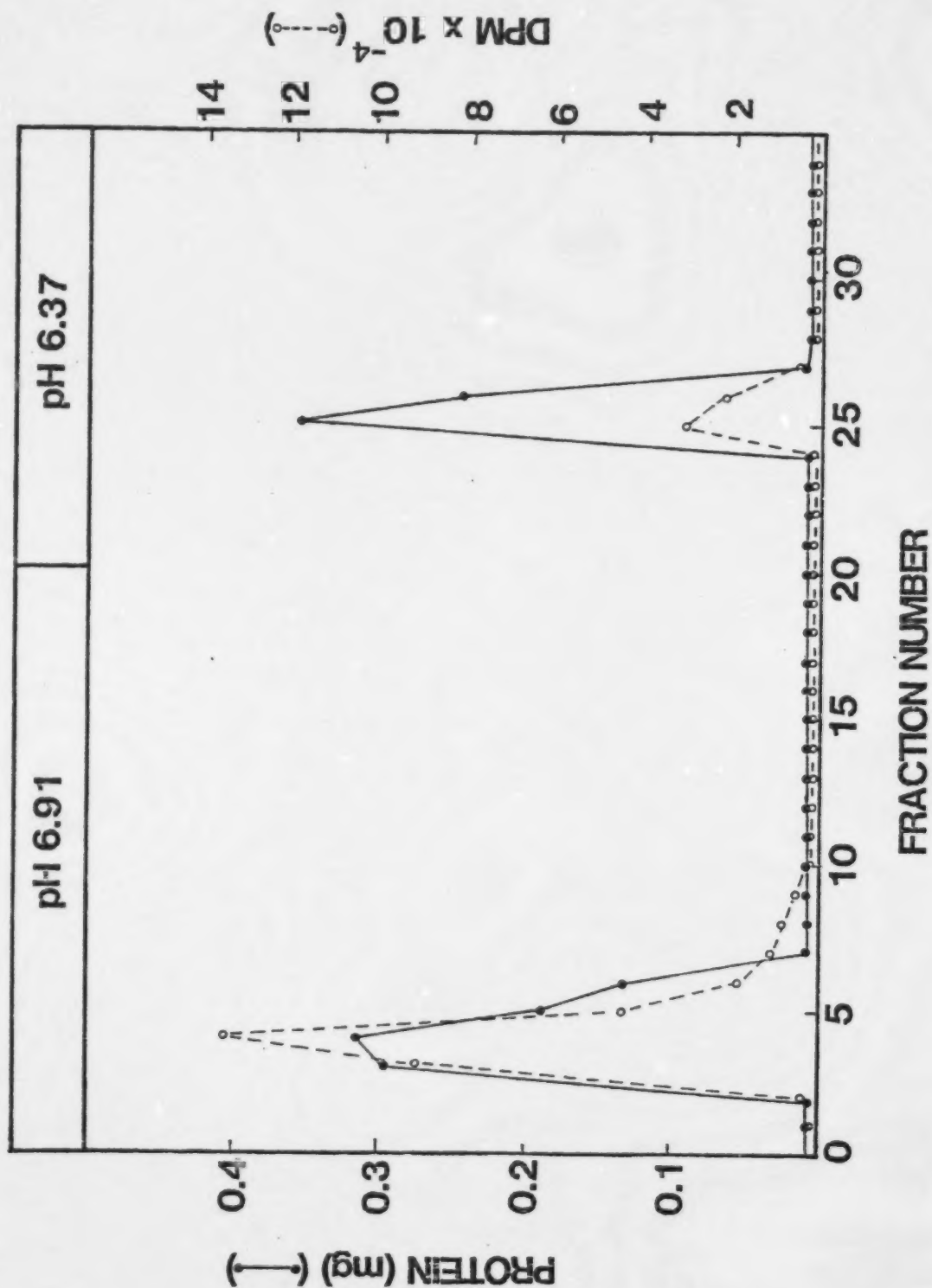


Figure 4a.

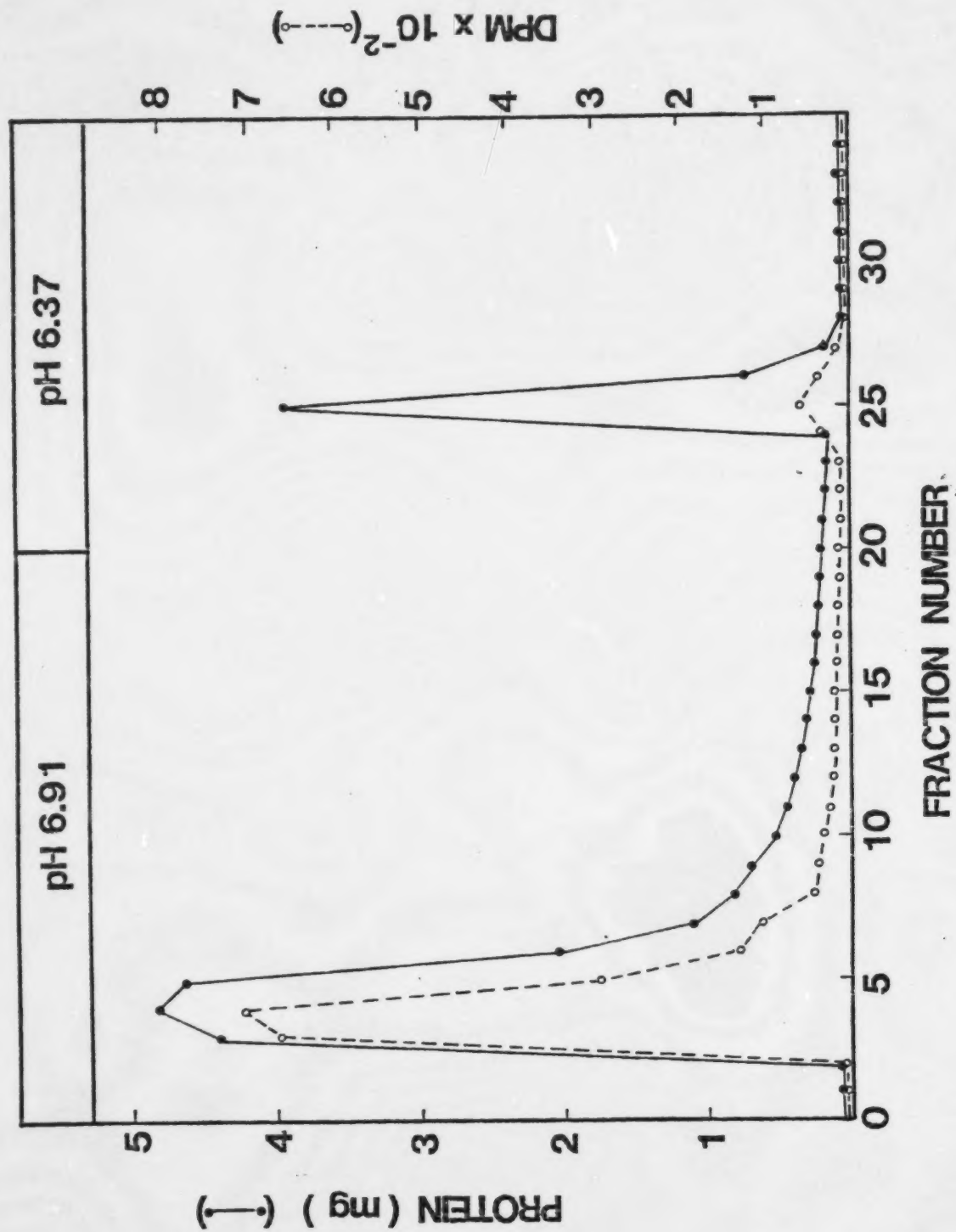


Figure 4b.

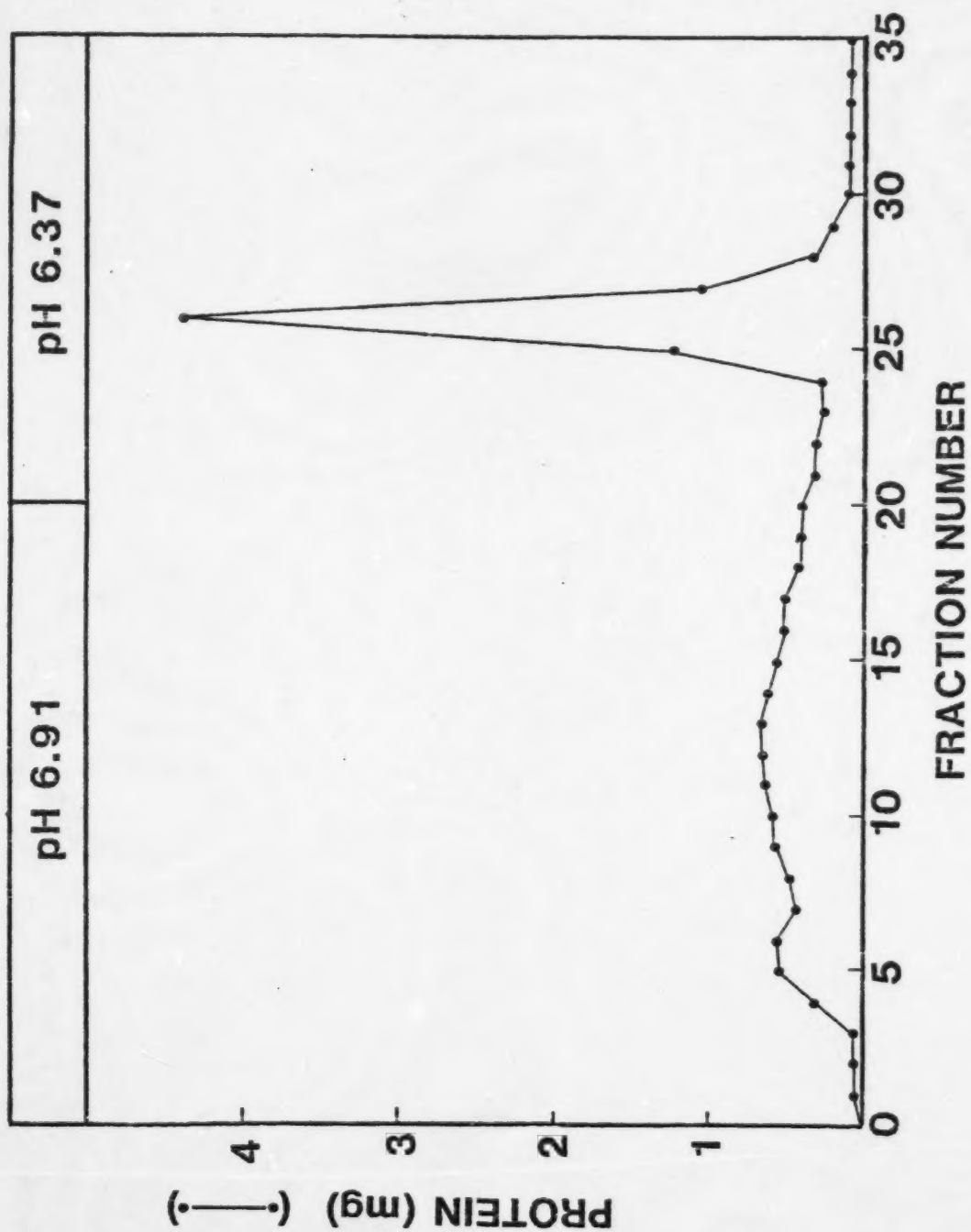


Figure 4c.

eluted in four peaks distinct from the amino acids (Figure 5). Therefore, the radioactivity present in the hemoglobin represented adducts to amino acids of a reactive metabolite of chloroform. Thee reactive metabolite of chloroform has been demonstrated to be phosgene (Pohl, 1979).

CONCLUSIONS

Hemoglobin binding of chemical carcinogens was demonstrated to possess the following attributes of a dose monitor: a) the extent of carcinogen binding to hemoglobin was dose related and b) in circulating erythrocytes, the hemoglobin containing bound carcinogen was as stable as normal hemoglobin so that hemoglobin binding could integrate the daily binding that occurred over the long life time of the erythrocyte. This increases the sensitivity of the dose monitor to chronic low level doses such as those that result from environmental exposure.

A procedure is being developed for the isolation of carcinogen adducts in hemoglobin that employs as its initial step the isolation of the hemoglobin containing the bound carcinogen.

Fig. 5

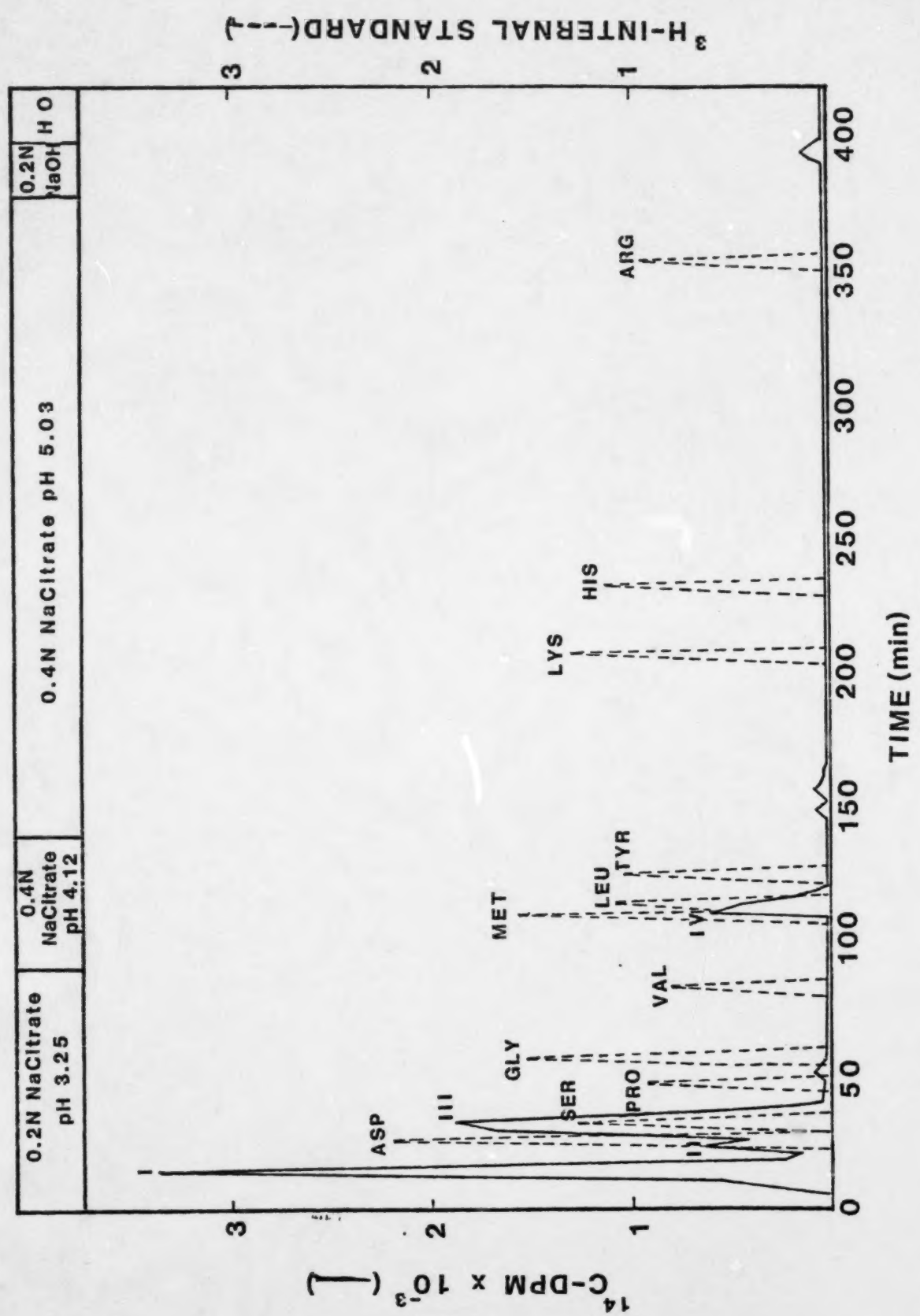


Figure 5.

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